Evaluating how Fish Oil, with a concentration of 0.03%, treatment affects bovine luteal cells in a hypoxic environment

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The Corpus Luteum(CL) is an endocrine gland that grows temporarily on the ovary when an ovum is released. This gland secretes the steroid hormone progesterone, which is vital to the maintenance and establishment of pregnancy in bovine. Prostaglandin (PGF2a) causes pregnancy termination and failed regulation of PGF2a often leads to early embryonic mortality. (15) Considering that maternal PGF2a secretion results in both a structurally and functionally regressed CL, there is an inverse correlation as PGF2a secretion increases CL decreases to below 4 um. Therefore, reducing or eliminating the luteal cells sensitivity to PGF2a should decrease the amount of early embryonic mortality rates as well as CL regression.

Progesterone begins as cytosolic lipid droplets. It enters into the mitochondria of the luteal cell through an enzyme called STAR D1. Once inside the mitochondria, another the CYP 11 A1 enzyme converts the cholesterol into Pregnenolone , which leaves the mitochondria and travels to the endoplasmic reticulum (ER), where the enzyme 3BETA-HSD converts it into progesterone. (8)
Refer to the following figure.

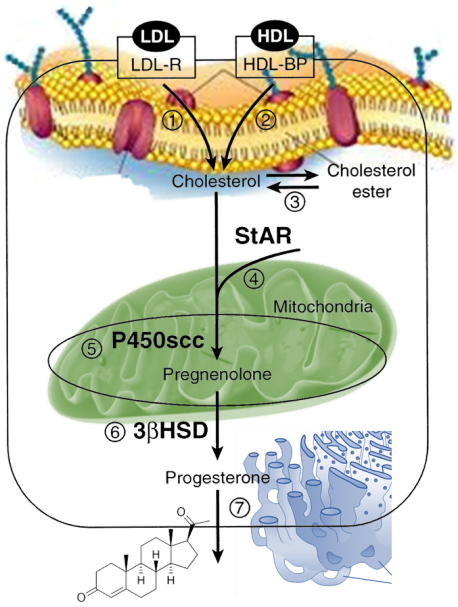


Figure 1

The potential of mitochondria membrane will be measured by confocal microscopy (better described as laser confocal scanning microscopy) and flow cytometry. This method of microscopy is used to garner a clearer optical resolution and contrast of a micrograph by using a spatial pinhole to block out

the out of focus light images. This depicts a brighter and more appealing image. While Confocal Microscopy is used to create more intense images of subjects, Flow Cytometry is used as a cell sorter that produces graphs. Flow Cytometry captures cells individually through a single cell suspension and reflects light off of them to see how well stained they are, what their size is, and how intricate the cells actually are. The different options for the graph are side scatter and forward scatter, depending on how the light reflects off the cells. (6)

As an important source of food, byproducts, and because they reduce the chance of wildfire, the cattle (bovine) industry is one of the world's most important agricultural enterprises.To improve the industry, we need to improve the rate of maternal recognition, the rate at which female bovine detects a viable pregnancy and does not terminate, of pregnancy and decrease the rate of miscarriages. In bovine, the maternal recognition rate is very low- 40% in dairy cows and 65% in beef cows, and each lost pregnancy costs the producer $2,300(9).

In bovine, failure of maternal recognition or loss of pregnancy is likely to be caused by Prostaglandin. Prostaglandin acts through a signal transduction pathway to decrease the rate of transcription of the STAR D1, Cyp 11-A1, and 3B-HSD enzymes, ultimately this results in a reduction of progesterone production (functional regression), and in size (structural regression) (12).

Previous studies out of Dr. Burns' lab describe the effects of Omega 3 long chain fatty acids on ovarian and uterine function as beneficial. "There are three omega 3 fatty acids that can have a significant impact on cellular physiology and possibly reproduction: α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)." (2) Because of these positive effects, we are hoping to reduce the impact of prostaglandin on the corpus luteum. In humans, these positive effects include prevention of pre-term labor, lower the risk of preeclampsia, and possibly increases birth weight.(16) This study is hoping to prove that omega three fatty acids also increase the mitochondrial membrane's defense capabilities against prostaglandin.

In addition to other negative effects, Prostaglandin affects corpus luteum function through decreasing the blood flow, inducing hypoxic conditions.(8) Hypoxia offers the simplest way to replicate the effects of prostaglandin in a laboratory environment. This study's purpose is to recreate this hypoxic

environment in two experimental groups and compare those to two control groups. The first control will be in a 20% oxygen chamber treated with vegetable oil, while the first experimental will be in a 20% oxygen chamber and treated with fish oil.

Although treated with different supplements the results should be the same in both groups, each should be equally healthy, this is not the case in the second control and experimental. The second control will be in a 1% oxygen chamber and treated with vegetable oil, while the second experimental will also be in a 1% oxygen chamber and treated with fish oil. The control group will be very unhealthy, while our experimental group should be closer in health to the 20% oxygen groups.

The hypothesis is while growing under hypoxic conditions the bovine luteal cells will demonstrate greater mitochondrial membrane potential when treated with fish oil as opposed to those treated with vegetable oil (control). To the contrary, the null hypothesis is when bovine luteal cells are treated with fish oil under hypoxic conditions their mitochondrial membranes will not demonstrate greater potential. This hypothesis is based upon previous studies indicating that fish oil has protective properties and alters treated cells membranes.(8) Overall, when these protective properties begin to go into effect it will increase the mitochondria's membrane potential which directly correlates with the rate of steroidogenesis and health of the mitochondria. (8)

Methods

For the purposes of our investigation, ovaries were collected from the Greeley slaughter house and transferred to University of Northern Colorado Greeley in 1x PBS. When the ovaries arrived at the university the Corpus Luteum (CL) was dissected away from the ovary. The CL was cut into 1 mm slices and placed in a solution called Hank's Balanced Salt Solution(HBSS). Then the samples were moved to T75 culture flasks and incubated in a rocking water bath at 37°C with type 1 collagenase and Bovine Serum Albumin (BSA). The samples then incubated for a few hours in a rocking water bath at 37°C. After incubation the samples were pushed through a filter to separate the larger debris, such as connective

tissue. Then the samples were spun down in a centrifuge at 500 G, and transferred to T75 culture flasks and resuspended in HAMS-F12 media, FBS, insulin transferrin serum, and antiviral-antibacterial supplements (Anti Anti).(8)

Cells were then prepared for flow cytometry by removing the culture media from the T75 culture flasks. Each flask was washed two times with 1x PBS. After that, approcimately 4 mL of trypsin is added to each flask, then the cells incubated with the trypsin at 37°C for 15-20 minutes. The trypsin and cell mixture was then pipetted into a 15 mL conocal tube and spun down in a centrifuge at 500 G for 5 minutes. The supernatant was removed and the pellet is suspended in 1% BSA-PBS. The cells were divided into microcentrifuge tubes according to their corresponding treatment groups. The first treatment group received 1% PBS and BSA, while the second treatment group received TMRM (tetramethylrhodamine, concentrated at 100 nanomolar) added to 1% BSA. Finally, the third treatment group was incubated with CCCP (Carbonyl cyanide m-chlorophenyl hydrazone with a 50 mM stock concentration) to form the negative control group. The cells treated with CCCP then incubated at 37°C for five minutes, after which TMRM was added to them. (8)

Specific groups were ran for our Confocal Microscopy, including a 20% oxygenated environment where there were two four-well plates and a 5% oxygenated environment where there were also two four-well plates. Each plate in each hypoxic environment contained a vegetable oil treatment group, a BSA control treatment group, a CCCP treatment group, and a fish oil treatment group. A cell sorter was utilized to deliver exactly 12,500 cells into each well. (8) See figure below.

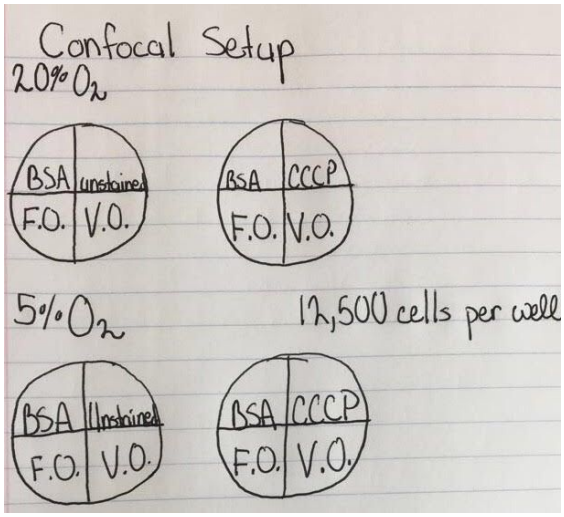


Figure 2

The vegetable oil and fish oil were bound to BSA by incubation in a rocking water bath for two hours at 37°C before being applied as a treatment. After being bound they were then applied to their specific groups. CCCP had not yet been applied. These cells were then incubated for three days at 37°C and then grown in either 20% oxygen or 5% oxygen for 24 hours. At hour 24 of this study 0.5 microliters of TMRM was applied to the BSA control, fish oil, and vegetable oil groups. Then the CCCP was applied to it's designated cells. After this the cells were observed under the confocal microscope. (8)

While we were running tests on our treated cells, there was also a study being done on progesterone production using Forskolin as an activator in hypoxic conditions. Six six-well dishes were plated out with 12,500 cells in each well. Half of the wells were treated with 1.64 microliters of Forskolin DMSO, while the other half were treated with BSA control, BSA bound fish oil, and BSA bound vegetable oil. Forskolin was used, instead of something like Luteinizing Hormone(LH), which is the hormone that is released after estrus to cause follicle rupture and the release of an ovum, because of it's properties as an activator of adenylylcyclase. Adenylylcyclase activates cyclic adenosine monophosphate (cAMP) which in turn activates the enzyme STAR and increases it's production. Without this STAR enzyme cytosolic lipid droplets, chloresterol, would never be able to enter the mitochondria and in turn become progesterone. These treatments were then left on the cells for three days. (8)

After this three day period, cells were grown at either 20% or 5% oxygen for 24 hours.

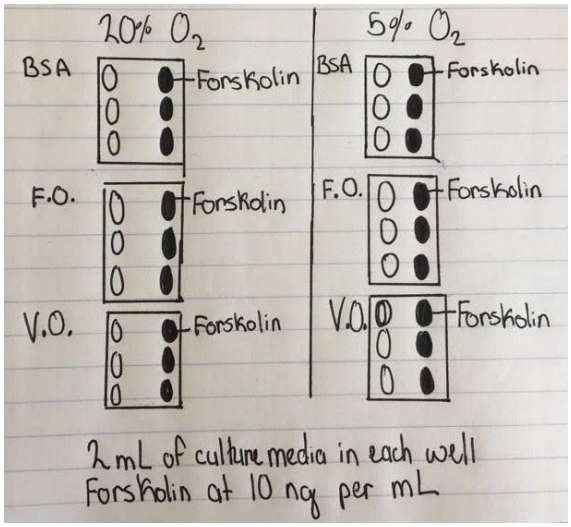


Figure 3

During that time 50 microliters of culture media was taken from each well at the exact time points of 0 hours, 6 hours, 12 hours, and 24 hours. At hour 24, DNA was extracted from each well. This was done by taking off the culture media from each well, then the wells were washed three times with 1x PBS. 100 microliters of tryzol reagent was then added to each well, and the cells were scraped with a cell scraper. After being scraped, the cells were collected and put into epi tubes and frozen at -80°C.(8)

Results

Unstained Cells

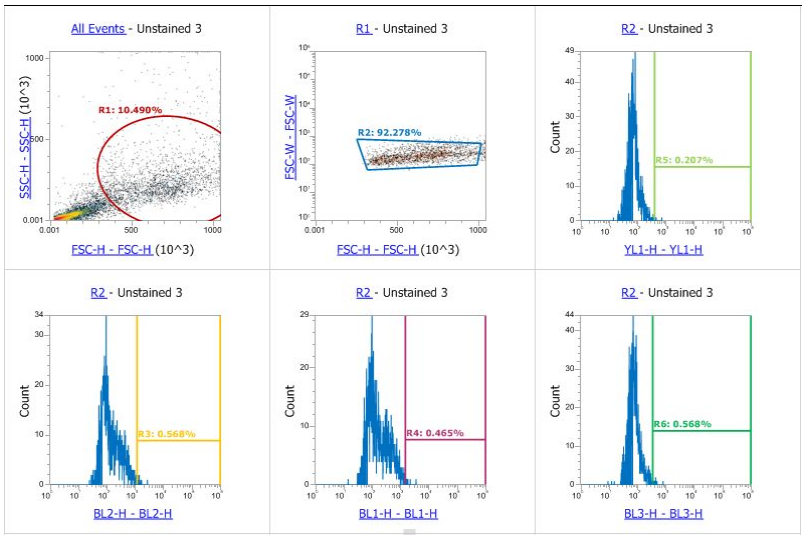


Figure 4

Depicted above in Figure 4 is the first assay that represents data collected from the unstained cells. In the box labeled All Events is a density plot that reads how complex the innerworkings of the cells are as well as how large they are. Inside of the red circle shown on this graph is a population of cells comprised of large and internally complex cells which are characteristics of Luteal Cells. R1 graphs this specific population and is gated to account for doublets. Doublets occur when two cells are ran through flow and incorrectly sorted as large and complex cells. In the remaining graphs, the population within the blue gate of R1 are shown. R2-YL1 portrays the flourescence at 568 nanometers. All of the cells, except for unstained, were stained with TMRM, which excites at 488 nanometers. However, this graph is plotted in terms of 568 nanometers. Considering this is a graph with unstained cells there should be no cells within the gate for R2- YL1. Regarding the rest of the samples, there should be no cells within the gates of R2-BL2, R2-BL1, or R3-BL3.

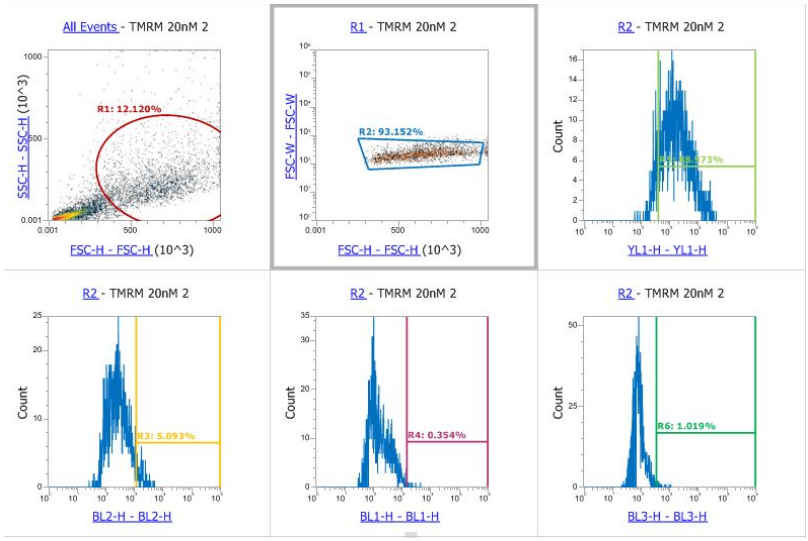


Figure 5

Depicted in this assay are the TMRM stained cells. As with the previous assay, R1 is based off the gate of All Events, and the rest of the graphs are based off of the gate within R1. However, there is a noticeable differentiation between R2-YL1 of these TMRM treated cells compared to the Unstained Cells. The TMRM stained cells have 88.973% of their cells being flourescent, as compared to 0.207% for the Unstained.

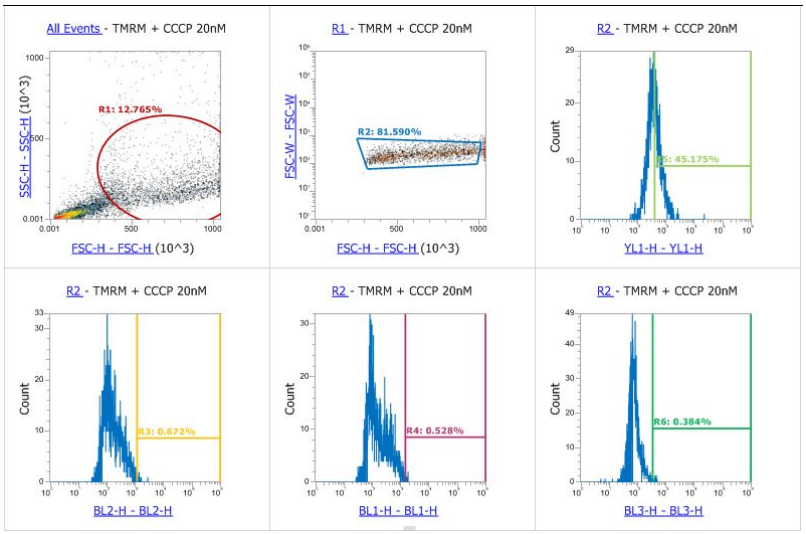


Figure 6

In this third assay, the cells portayed are those treated with CCCP along with being died with TMRM. As with the previous assay, R1 is based off the gate of All Events, and the rest of the graphs are based off of

the gate within R1. However, in R2-YL1 there was a decrease in cells that showed being within the flourescence perimeter. In the TMRM treated cells 88.973% of the cells showed flourescence, as opposed to the 45.175% of the CCCP treated cells. This can be interpreted as a significant drop in cells that are alive versus dead.

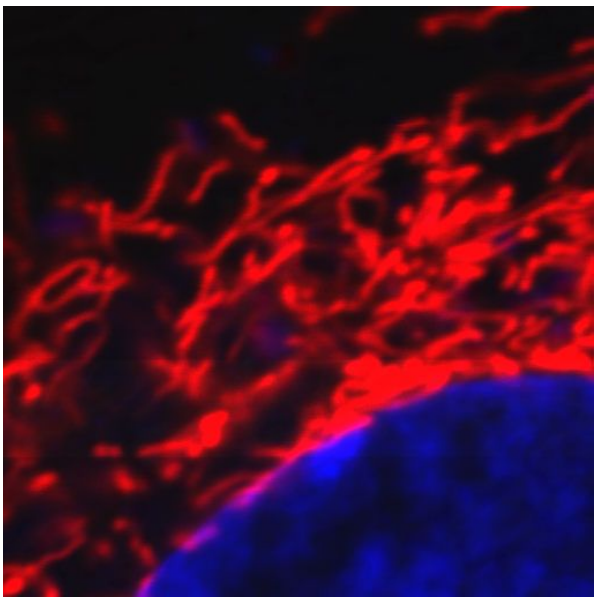


Figure 4

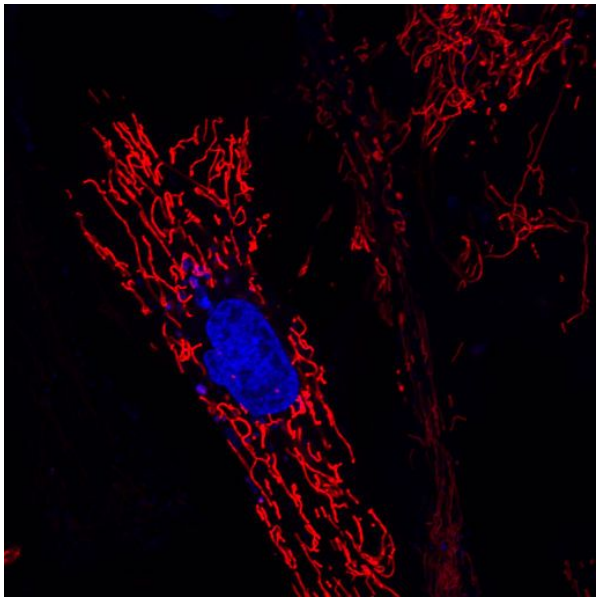


Figure 5

Above are pictures taken from a Confocal Microscope. They are images of the BSA control. Figure 4 is fragmented whereas Figure 5 is unfragmented. Fragmentation refers to the shape of the mitochondria. Mitochondria are supposed to be long rod shaped organelles, as depicted in Figure 5. However, when fragmentation occurs small pieces of the organelle break off and take on a circular form, as depicted in figure 4. The blue stain within the picture is Hoechst's Dye, this dye targets the nucleus and stains it bright blue. TMRM stains the mitochondria red. The greater the mitochondria's potential the more intensely the red flourescence appear

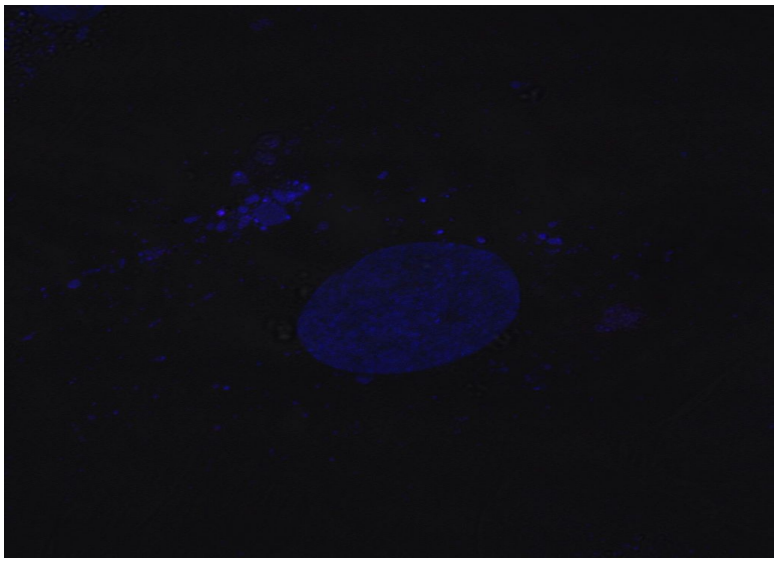


Figure 6

In Figure 6 is a cell treated with CCCP then dyed with TMRM. This depicts a very unhealthy cell with a strong lack of intensity in the dyes. This is expected because CCCP should have a negative effect on cells.

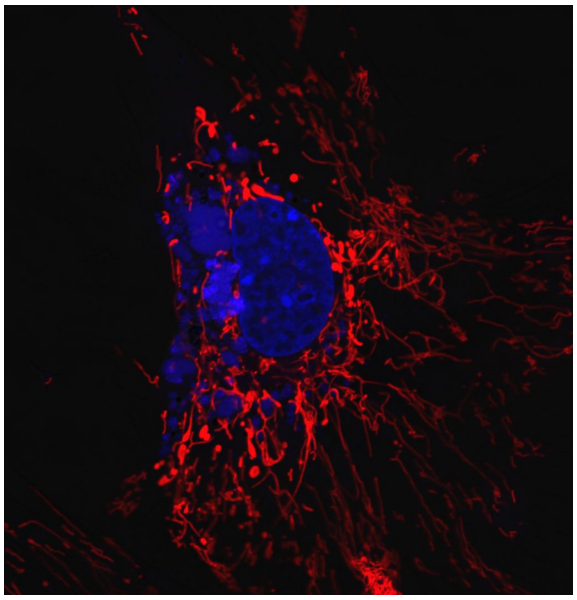


Figure 7

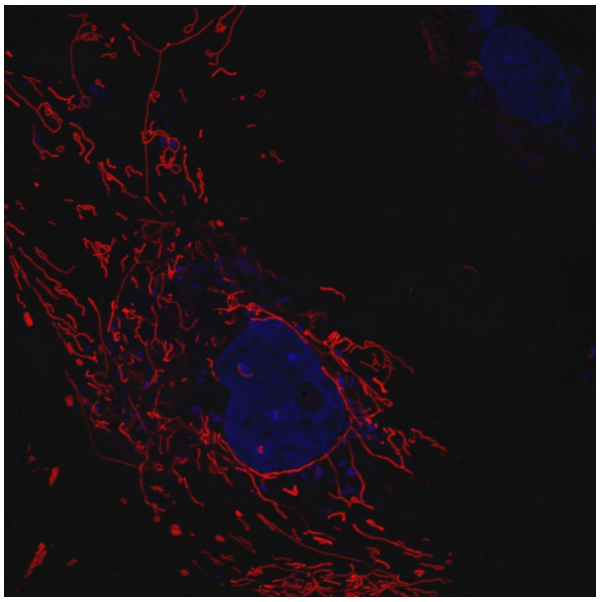


Figure 8

Figure 7 portrays cells treated with Fish Oil and then dyed with TMRM. While Figure 8 portrays cells treated with Vegetable Oil and then dyed with TMRM. Both cells have very high intensity mitochondria, this indicates high mitochondrial membrane potential.

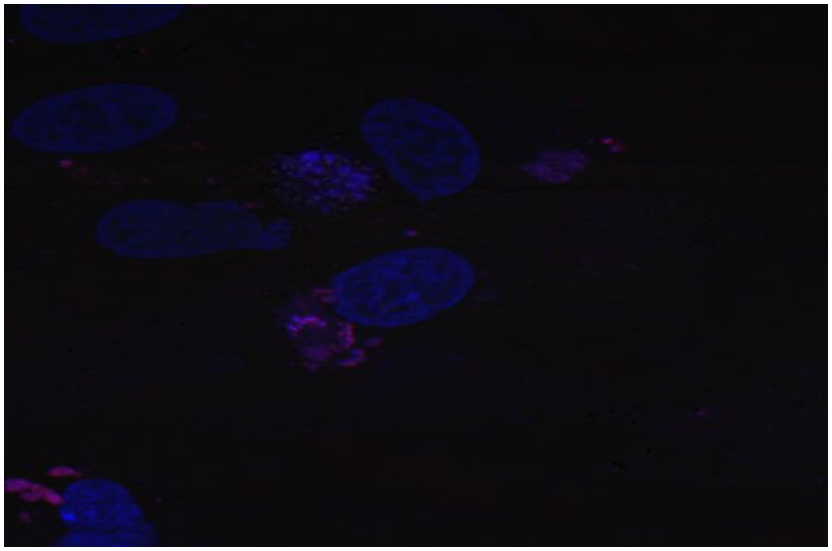


Figure 9

Above, depicted in Figure 9, are unstained cells. These serve as a negative control variable to compare the stained cells with.

Results of Hypoxia at 5%:

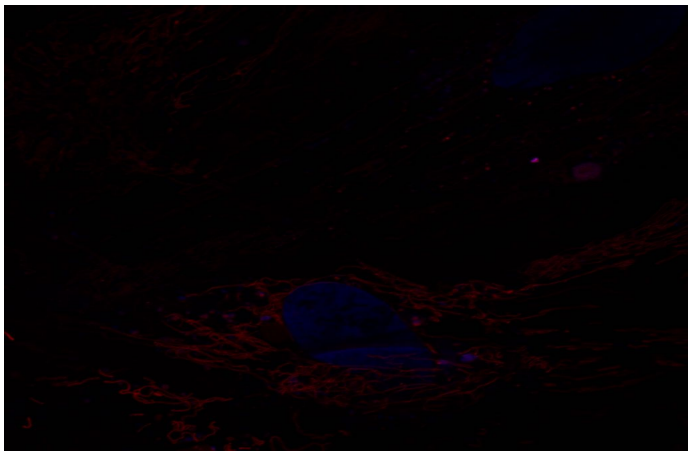


Figure 10

Figure 10 is a cell treated with BSA then TMRM. The lack of TMRM flourescence intensity indicates that the mitochondria have become depolarized due to the lack of oxygen.

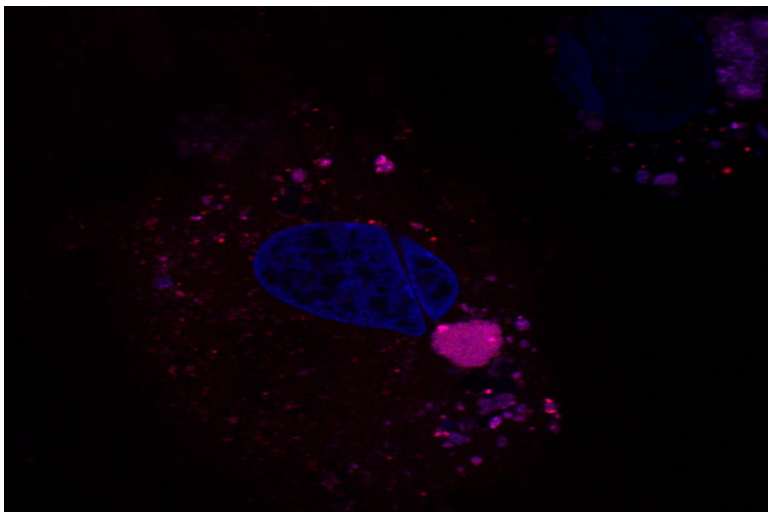


Figure 11

Figure 11 is a cell treated with CCCP then TMRM. Correlating with the image before Figure 11, there is an obvious lack of flourescence.

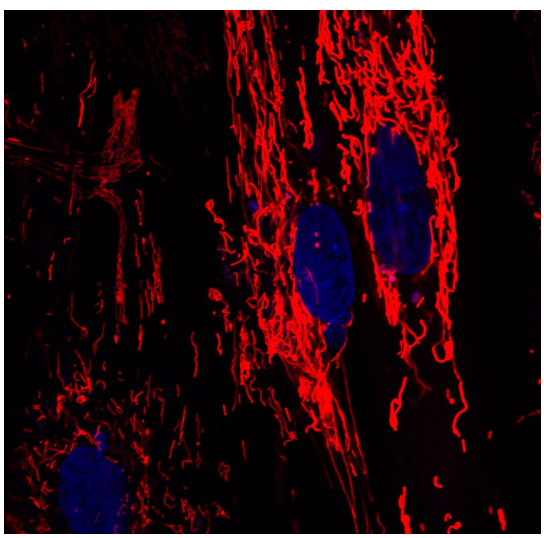


Figure 12

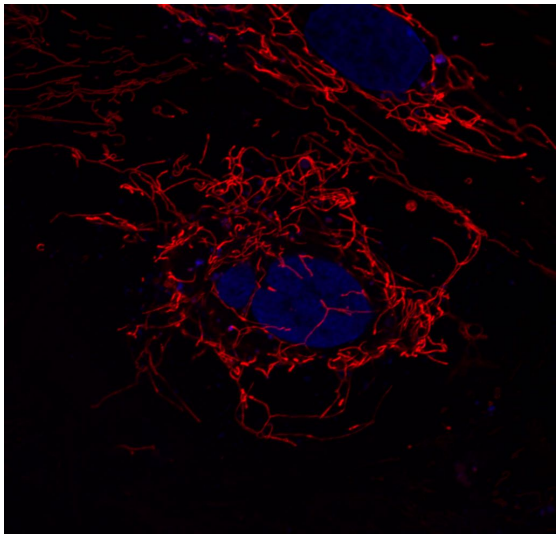


Figure 13

Figure 12 depicts cells treated with Fish Oil and then with TMRM. In contrast to other cells there is a vivid flourescence, indicating that the cell is alive, and the mitochondria are polarized. Figure 13 depicts cells treated with Vegetable Oil and then with TMRM. In comparison, to the Fish Oil treated cells, the

Vegetable Oil treated cells are slightly less polarized and are seen with a lack of order. Also, although the Fish Oil treated Cells do depict flourescence they are not as vivid as the Fish Oil treated cells.

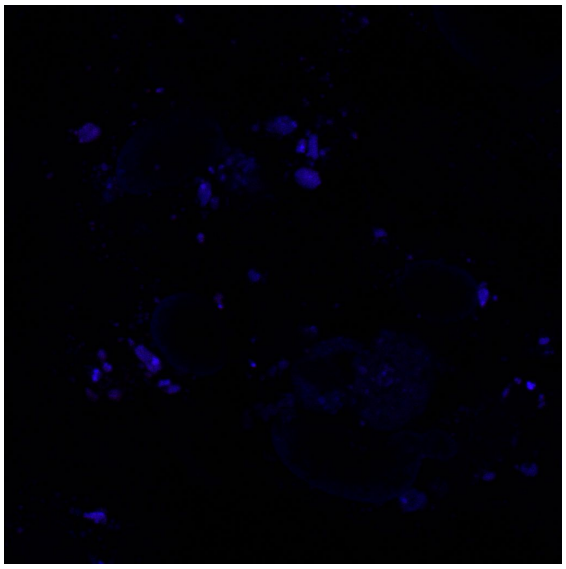


Figure 14

In Figure 14, the last of the 5% oxygenated cells, Unstained Cells are shown. These cells do not have any fluorescence because they were not exposed to the TMRM dye as the other cells were.

Cells grown in 20% Oxygenated Environments:

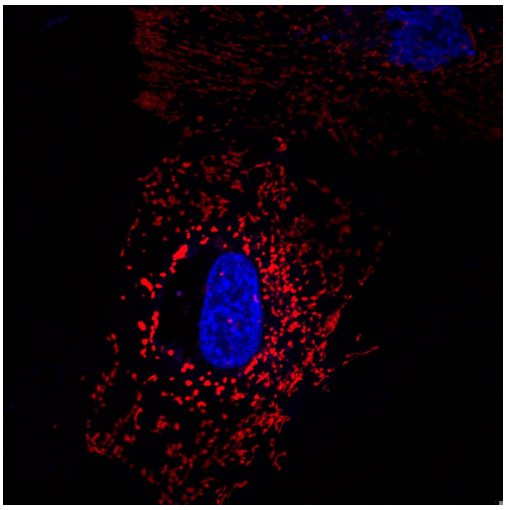


Figure 15

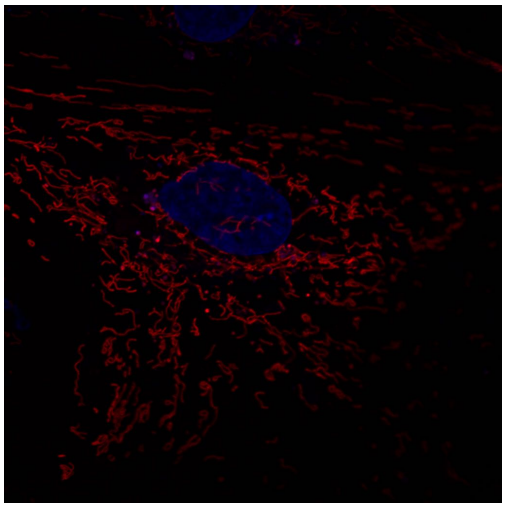


Figure 15

Figure 16

Hour 24-20% Oxygen-CCC

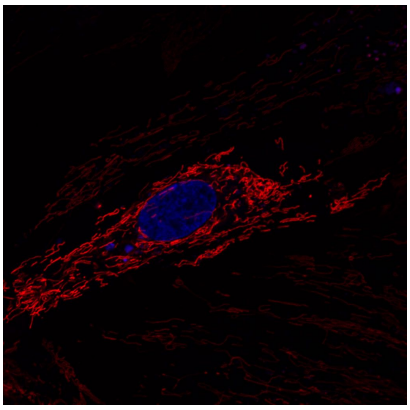


Figure 17

Hour 24-20% Oxygen-Fish Oil

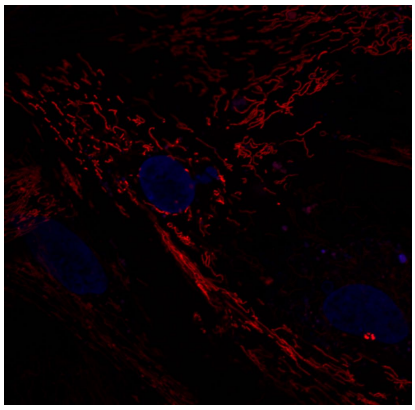


Figure 18

Hour 24-20% Oxygen-Unstained

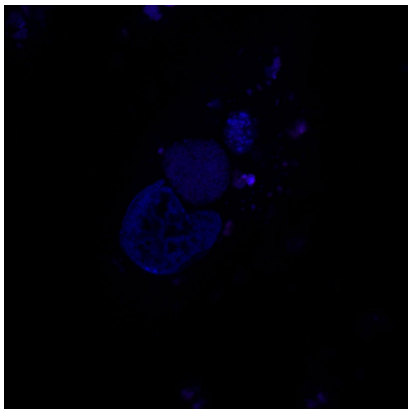


Figure 19

From the data that was collected from both the confocal microscopy and flow data we know the hypoxia did negative effects of the cells. Images of fish oil in 5% hypoxic conditions were brighter compared to BSA control and Vegetable Oil.It appears that the fish oil had protective affects based upon the amount of data collected.Vegetable oil also appeared to have a small affect but not quite as much as the fish oil did. Maybe because the fo had EPA and DHA

When we stained the mitochondria the Flow picked up a large colony of mitochondria. But with the cyanide a lot of the mitochondria of the cells dimmed. So we are confident that when the cells are unhealthy the die dims down.

Flow Cytometry Data:

CCCP treated Cells From the data that was collected from both the confocal microscopy and flow data we know the hypoxia did negative effects of the cells. Images of fish oil in 5% hypoxic conditions were brighter compared to BSA control and Vegetable Oil. Refer to the following figures.

BSA Control Hour 24 20% Oxygen

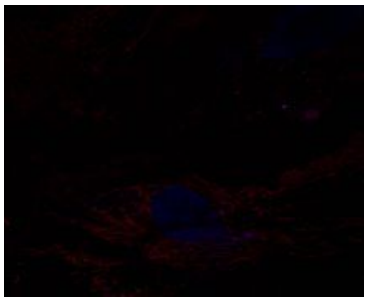


Figure 4

Fish Oil Hour 24 20% Oxygen

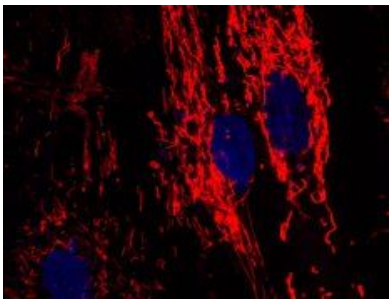


Figure 5

Vegetable Oil Hour 24-20% Oxygen

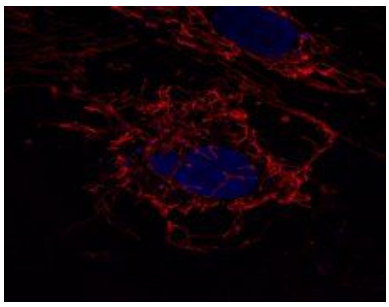


Figure 6

Discussion

From the data that was collected from both the confocal microscopy and flow data we know the hypoxia did negative effects of the cells. Images of fish oil in 5% hypoxic conditions were brighter compared to BSA control and Vegetable Oil. Refer to the following figures.

BSA Control Hour 24 20% Oxygen

Fish Oil Hour 24 20% Oxygen

Vegetable Oil Hour 24-20% Oxygen

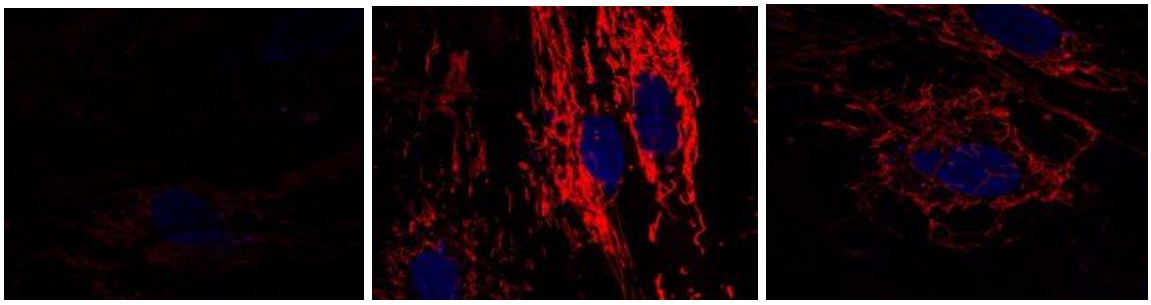
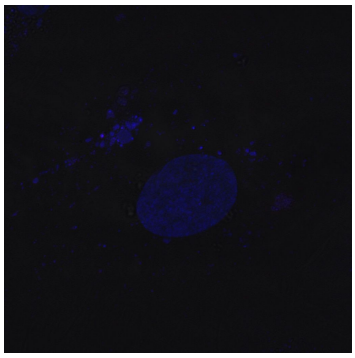


Figure 4 Figure 5 Figure 6

Also, when the Luteal Cells were stained, the flow picked up a large colony, but when the cells were treated with cyanide many of the stained cells dimmed. Meaning, they were no longer showing up as much. Because of these observations, it has been concluded that when the cells are treated with cyanide the stain dims and the cells are very hard to observe or process. Refer to the figures below.

Cyanide Treated Hour 0 Fish Oil Hour 0



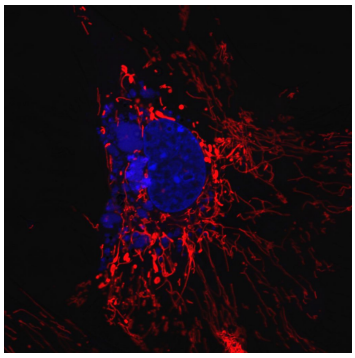


Figure 7 Figure 8

Overall, this experimental research is deemed inconclusive in all aspects except for one. It appears as though there has been a positive effect on cells treated with fish oil, even in hypoxic conditions, yet, there is not enough data to state this as a fact. Therefore as of right now, fish oil is proposed to have positive effects against the

RESULTS

The hypoxia had negative effects of the cells.

Images of fish oil in hypoxic conditions were brighter compared to BSA control/Vegetable Oil.

Discussion

Go in more depth.

It appears that the fish oil had protective affects based upon the amount of data collected.Vegetable oil also appeared to have a small affect but not quite as much as the fish oil did. Maybe because the fo had EPA and DHA

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References

(1) Plewes, M. R., Burns, P. D., Hyslop, R. M., & Barisas, B. G. (2017). Influence of omega-3 fatty acids on bovine luteal cell plasma membrane dynamics. *Biochimica Et Biophysica Acta (BBA) - Biomembranes,1859*(12), 2413-2419. doi:10.1016/j.bbamem.2017.09.012

(2) Plewes, M. R., Cedillo, J. C., Burns, P. D., Graham, P. E., Bruemmer, J. E., & Engle, T. E. (2018). Effect of fish meal supplementation on luteal sensitivity to intrauterine infusions of prostaglandin F2alpha in the bovine†. *Biology of Reproduction*. doi:10.1093/biolre/ioy003

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